Plant Growth Regulation **30:** 225–232, 2000.

Effects of early plant growth regulator treatments on flavonoid levels in grapefruit

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Received 8 September; accepted 7 October 1999

Key words: abscisic acid, *Citrus paradisi*, flavonoids, foliar sprays, gibberellic acid, grapefruit, juice, juice sacs, naringin, narirutin, peel, reduction

Abstract

Early foliar spray treatments containing gibberellic acid (GA_3) significantly lower the concentration of the bitter flavonoid naringin in fruit tissues. Sprays containing a surfactant and different levels of GA_3 (5, 50, 100, and 500 ppm) or abscisic acid (ABA) (5, 25, and 50 ppm) were applied to young, developing fruit on mature grapefruit (*Citrus paradisi*) trees during the period from April to June, beginning just after fruit set. The fruit were allowed to mature and were harvested early the following year. Harvested fruit were evaluated for weight, juice characteristics, and flavonoid concentrations. GA_3 application resulted in larger mature fruit, which yielded juice with the same soluble solids value as juice from control fruit, but with slightly lower acid percentages and lower concentrations of naringin. ABA treatment had little effect on juice soluble solids, acid content and naringin content except at the highest concentration of 50 ppm, which lowered naringin levels slightly in juice.

1. Introduction

Application of plant growth regulators (PGRs), especially but not exclusively gibberellic acid, to citrus trees to enhance fruit yield and quality has been performed on commercial crops for a number of years [16, 48, 49]. The PGRs have also been used to alter flowering, fruit set, fruit thinning and fruit abscission [18], to treat certain fruit diseases such as corky (silvery) spots [22], and to reduce fruit susceptibility to attack by the Caribbean and Mediterranean fruit flies [23, 38]. Although some research has been published on the application of auxins, cytokinins, and abscisic acid (ABA) to developing citrus fruit [18], the bulk of the research has dealt with the application of gibberellins, mostly in the form of gibberellic acid A_3 (GA_3). Preharvest GA_3 application delays some of the ripening processes such as color break (degreening), slows rind softening, and under certain conditions prevents some rind blemishes. The application process has been studied extensively by the research groups of Goldschmidt and Coggins [16, 20, 21, 24–27]. Acidification and the addition of wetting agents enhance the uptake of GA_3 by citrus fruit. However, the mixture of certain wetting agents and GA_3 can cause rind damage [15]. There is some evidence that these PGRs could be used to alter the accumulation of sugars, acids, and especially flavor components [18]. Two previous studies have shown that application of GA_3 can lower the levels of the bitter flavonoid naringin in grapefruit [12, 45].

The four flavanone neohesperidosides – naringin, neohesperidin, neoeriocitrin, and poncerin – are found in citrus species related to the pummelo (*Citrus grandis* (L.) Osbeck) and cause flavonoid bitterness

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in economically important species such as the sour orange and the grapefruit [9, 35, 37]. Pummelo accumulates the flavanone neohesperidosides exclusively, while sour orange (*Citrus aurantium* L.) and grapefruit accumulate both the flavanone neohesperidosides and the tasteless flavanone rutinosides found in other citrus species related to the citron (*Citrus medica* L.) and the mandarin (*Citrus reticulata* Blanco) [2]. The taste properties of the flavonoid glycosides are determined by the sugar linkage as well as some other factors [34]. Several detailed studies on naringin content in grapefruit and sour orange have been published [1, 37, 45], while there has been only two reports on flavonoid composition and concentrations in pummelo [9, 37]. Naringin is by far the most abundant flavonoid bitter principle in grapefruit [2, 30] and pummelo [9, 35, 37], while neohesperidin is slightly more abundant in sour orange [13]. Neoeriocitrin and poncirin only occur in relatively minor amounts in juices. Naringin levels have been found to vary from over 600 ppm in the early season to below 100 ppm in the late season in some grapefruit varieties [3, 4, 7, 30]. The taste threshold for detection for naringin is approximately 20 to 50 ppm. Comprehensive studies on naringin taste thresholds were conducted by Guadagni et al. [28, 29].

The pathway of flavonoid biosynthesis in citrus is similar to that in other plant species [32], and is highly regulated. Constitutive levels of flavonoids are produced during normal growth and development, but additional formation of specific compounds can be induced by wounding and attack by pathogens [46]. In citrus, the flavonoid glycosides accumulate in young leaves and fruits during the cell division stage [10, 11, 13, 14, 19]. The woody portions of the plant do not biosynthesize flavonoids, though these compounds are found in stem and root tissues [11, 36]. During cell elongation and subsequent maturation of leaves and fruit, there is little further biosynthesis. As fruit cells expand and reach maturity, flavonoid concentrations are lowered due to dilution effects [5, 14, 30, 36, 44, 47]. Thus juice from early season grapefruit is generally more bitter than juice from late season grapefruit [7]. Levels of naringin are much higher in the peel than in the juice vesicles, and because naringin is very soluble in water, the naringin content of commercially processed juice is dependent on the pressure applied to the fruit during the extraction process.

Application of PGRs can have an effect on the accumulation of these flavonoids, especially if applied to young fruit during the period of intense flavonoid biosynthesis. Both radiolabled GA_3 and benzylaminopurine (BA) have been shown to be taken up by the fruit [26, 27, 50]. Only the effectiveness of GA_3 and BA have been studied to any degree [18, 48]. Higher hesperidin levels were maintained for a longer period of time in Tangelo (*C. reticulata* × *C. paradisi*) fruit treated with BA [17]. The authors inferred that this could be due to either increased biosynthetic enzyme activity or increased translocation of the flavonoids into the fruit from other parts of the plant. Naringin levels were unaffected or lowered slightly by applications of GA_3 to fully expanded, pre-color break fruit [45]. The lack of effectiveness of this treatment may be due to the late GA_3 applications to mature fruit. Application of GA_3 in lanolin paste to immature fruit was shown to be effective at lowering the levels of naringin in both the peel and the juice [12]. However, BA in our earlier study was ineffective as a regulator of flavonoid accumulation, and was actually detrimental to fruit development. On the basis of our previous study, and some unpublished observations in our lab that ABA promoted the accumulation of unique flavonoids in citrus cell culture studies, we evaluated the spray application of GA_3 and ABA to immature 'Duncan' grapefruit to determine if the levels and quantities of the flavonoids could be affected by these treatments.

2. Materials and methods

2.1 *Chemicals*

Gibberellic acid A_3 (GA_3), 2-cis-4-trans-abscisic acid (ABA), and Triton X-100 were purchased from Sigma Chemical Company, St. Louis, MO. Flavonoid standards were either from the Robert M. Horowitz collection (now housed at the USDA, ARS, Citrus and Subtropical Products Lab, Winter Haven, FL), purchased commercially, or isolated from citrus sources. All other chemicals were purchased from local chemical supply firms.

2.2 *Grapefruit trees*

A single row of 20 year old grapefruit trees (*Citrus paradisi* (L.) Macf. c.v. 'Duncan') maintained at the University of California Citrus Research Center and Agricultural Experiment Station in Riverside, California, were used in these experiments.

2.3 *PGR treatments*

Two types of applications were used.

GA #1 was a spray application over an entire tree with a commercial sprayer provided by the Department of Botany and Plant Sciences, University of California at Riverside. A control and three concentrations of GA3 (5, 25, and 100 ppm) were each applied to four different trees in 0.1% Triton X-100 wetting agent until the entire tree was wet. The first application was on June 10, 1993, followed by a second application on July 1.

GA #2 involved the use of a hand-held pressurized sprayer containing differing levels of PGRs in 0.1% aqueous Triton X-100 on marked branches of individual trees, one tree for each experimental application. The marked branches were treated 5 times every 2 weeks starting on May 1, 1993. The marked branches were treated with 5 , 50, and 500 ppm GA3, and a second set treated with 5, 25, and 50 ppm ABA. The controls were treated with 0.1% Triton X-100. Untreated controls were also picked from the same trees.

2.4 *Sample preparation*

Mature fruit from the experimental trees were harvested in February 1994. The fruit from each experimental group were weighed. For the detailed flavonoid analysis, 30 fruit were selected from the samples and cut in half. Approximately a 2 gram portion of the peel and a 2 gram portion of the juice sacs from one segment were removed. Juice was prepared from the remainder of the half. After the preparation of the individual samples, juice was extracted by hand from the remainder of entire set of each treatment group fruit and pooled. A 50 ml portion of that mixed juice was saved for further analysis.

2.5 *Determination of juice soluble solids (BRIX) and acid percent*

The representative juice samples from each of the experimental groups were analyzed as done previously [12]. The juice samples were clarified by centrifugation. The total soluble solids were determined by refractometry. The acid percent was determined by titration of an aliquot of juice against a standardized solution of sodium hydroxide. Each sample evaluation was done in triplicate.

2.6 *Determination of flavonoid concentrations*

Samples were analyzed for flavonoid content by high-performance liquid gradient chromatography using a reverse phase column [9, 37]. Freeze-dried peel and juice sac samples were ground to a powder with a mortar and pestle with some added acid-washed sand, and extracted 4 times with a 50:50 mixture of dimethylsulfoxide and methanol. The extracts for each sample was combined and an aliquot filtered through a 0.45 micron filter for HPLC analysis. An aliquot of each juice sample was thoroughly mixed 1:1 with DMSO, centrifuged, and filtered through a 0.45 micron filter. HPLC analysis was performed on a dual pump HPLC system through a Licrosphere C18 reverse-phase column (4.6 $\times$ 250 mm, 5 micron, Phenominex Corp., Torrance, CA) equilibrated in 20% methanol 80% 0.01M phosphoric acid. An aliquot of sample was injected and the column was developed with a linear 20% to 100% methanol gradient at a flow rate of 1 ml/min. over a period of 50 minutes. Chemical peaks were detected by a photodiode array detector monitoring at 285 nm.

Individual flavanones and flavones were identified by confirmation of their unique spectra and retention times with those of authentic standards. Flavonoids were quantified using extinction coefficients determined from standard curves generated on the same HPLC system at 285 nm using purified naringin for flavanones, purified rhoifolin for flavones, and purified rutin for flavonols [9, 37].

3. Results

It has been previously reported that spraying trees with gibberellic acid caused leaf drop [49]. This did not occur with our treatments, which were done in May and June, even with the relatively high levels of GA_3 that were used. Most commercial GA_3 applications are done later in the season, resulting in stresses which cause leaf drop. Earlier season treatment does not seem to have the same effect. Fruit were allowed to mature on trees after treatment and were harvested in batches during February. In general GA_3-treated fruit were larger than untreated fruit. The peel of the GA_3-treated fruit was more mottled green than that of the controls, and there was some rind pitting and damage, as has been previously described for GA_3 used with wetting agents [15] though this was not severe. GA_3-treated fruit peel was generally thicker than that

Table 1. Juice analysis from Duncan grapefruits harvested in February 1994 from trees at the Riverside Agricultural Experiment Station. Mature trees were sprayed during May & June 1993. Fruit weights reported are means ± standard deviations. The juice was extracted and pooled from the entire number of fruit harvested in each treatment group. Analyses (BRIX, acid percent and naringin concentrations) were done in triplicate for each experimental group and the standard deviation for each set of analysis was less than 0.1%

Treatment	Experimental data				Juice quality		Naringin levels	
	Date harv.	# trmt	# fruit	fruit weight mean (g)	BRIX	Acid %	conc (ppm)	% of control
none	2/28	—	85	296.7 ± 54.3	9.2	1.45	382	100
Tween 80	2/21	4	101	260.1 ± 77.0	8.9	1.44	403	105
5 ppm GA-1	2/28	2	103	320.9 ± 65.8	9.2	1.51	324	85
25 ppm GA-1	2/28	2	96	338.8 ± 84.6	9.3	1.47	319	83
100 ppm GA-1	2/28	2	90	347.3 ± 59.6	9.3	1.45	238	62
5 ppm GA-2	2/21	4	82	324.9 ± 58.2	9.2	1.42	447	117
50 ppm GA-2	2/21	4	70	400.4 ± 76.0	9.1	1.28	374	98
500 ppm GA-2	2/21	4	68	432.5 ± 101.0	9.2	1.28	191	50
5 ppm ABA	2/15	4	43	263.0 ± 53.9	9.5	1.37	417	109
25 ppm ABA	2/15	4	103	261.0 ± 40.4	9.0	1.48	399	104
50 ppm ABA	2/15	4	71	396.9 ± 94.6	9.1	1.53	359	94

of control fruit, while segments from fruit representing both treatments seemed to be approximately the same size. The appearance of the ABA-treated fruit was similar to the control fruit. GA_3-treated fruit was generally larger than control fruit, while ABA-treated fruit were much more variable in size. The average size of the fruit treated with 50 ppm ABA was generally larger than that of control fruit, although there was a great deal of variability.

The juice analysis of the treated and untreated fruit is shown in Table 1. The Brix values were very similar, indicating that treatments had little effect on soluble solid levels in the juice. GA_3 treatments lowered acid levels in the juice. Naringin concentrations were lowered by all the GA_3 treatments, by as much as 50% in the case of 500 ppm GA_3, while ABA treatments had little effect.

Identification and quantitative analyses of the flavonoids were performed on peel, juice sac, and juice samples from 30 fruit in each treatment and control set. The results of this analysis for the major flavonoids present in the samples are presented in Tables 2, 3, and 4. Ten flavonoids were identified by their retention times and absorbance spectra relative to those of authentic standards. Naringin (naringenin 7-*O*-neohesperidoside), narirutin (naringenin 7-*O*-rutinoside), and, in the case of peel, poncerin (isosakuranetin 7-*O*-neohesperidoside) were found in the highest concentrations in these samples and are shown in the tables presented in this paper. In almost all cases, the GA_3 treatments above 5 ppm lowered the concentration of naringin in peel, juice sacs, and juice by statistically significant amounts.

In addition we were able to identify and quantitate naringin 4'-glucoside, apigenin 6, 8-C-diglucoside, hesperidin (hesperetin 7-*O*-rutinoside), neohesperidin (hesperetin 7-*O*-neohesperidoside), isorhoifolin (apigenin 7-*O*-rutinoside), rhoifolin (apigenin 7-*O*-neohesperidoside), naringin 6″-malonate, and didymin (isosakuranetin 7-*O*-rutinoside) which occurred at low levels (0.1 to 1 mg/g fresh weight or less) in our samples. In general, the flavonoids which occur in relatively low concentrations were unaffected by the treatments (data not shown).

4. Discussion

Previously it was shown that PGRs could alter flavonoid accumulation when applied to young fruit in a lan-

Table 2. Flavonoid composition in samples of peel from treated and control fruit (29–30 samples each). GA #2 are the individual fruit spray treatments, GA #2 are the whole tree spray treatments

treatment	flavonoid (mg/g fresh weight ± standard deviations)		
	narirutin	naringin	poncerin
control 2.15	6.52 ± 1.08	57.79 ± 7.65	2.65 ± 0.59
control 2.28	7.47 ± 1.49	57.02 ± 6.79	2.72 ± 0.84
tween control	4.98 ± 1.77	52.26 ± 9.95	2.32 ± 0.93
5 ppm GA_3 #1	5.14 ± 0.95	50.03 ± 6.88	2.12 ± 0.49
25 ppm GA_3 #1	4.72 ± 1.35	49.02 ± 8.13	2.17 ± 0.59
100 ppm GA_3 #1	3.45 ± 0.63*	40.11 ± 6.14*	1.67 ± 0.33*
5 ppm GA_3 #2	8.33 ± 4.11	61.65 ± 19.86	3.42 ± 1.53
50 ppm GA_3 #2	4.07 ± 0.84*	42.60 ± 7.10*	1.79 ± 0.37*
500 ppm GA_3 #2	2.97 ± 0.53*	37.50 ± 7.28*	1.49 ± 0.45*
5 ppm ABA	6.46 ± 0.87	56.00 ± 5.62	2.48 ± 0.41
25 ppm ABA	6.64 ± 1.21	55.69 ± 7.89	2.46 ± 0.47
50 ppm ABA	5.94 ± 1.39	52.60 ± 8.74	2.55 ± 0.78

* significantly lower than the tween-treated controls, according to student's t-test ($p < 0.01$).

Table 3. Flavonoid composition in samples of juice sacs from treated and control fruit (29–30 samples each). GA #1 are the individual fruit spray treatments, GA #2 are the whole tree spray treatments

treatment	flavonoid (mg/g fresh weight ± standard deviations)	
	narirutin	naringin
control 2.15	0.22 ± 0.07	0.41 ± 0.14
control 2.28	0.32 ± 0.11	0.59 ± 0.17
tween control	0.35 ± 0.15	0.55 ± 0.22
5 ppm GA_3 #1	0.26 ± 0.08	0.43 ± 0.14
25 ppm GA_3 #1	0.23 ± 0.06*	0.38 ± 0.09*
100 ppm GA_3 #1	0.21 ± 0.06*	0.38 ± 0.11*
5 ppm GA_3 #2	0.25 ± 0.09	0.41 ± 0.10
50 ppm GA_3 #2	0.15 ± 0.07*	0.20 ± 0.08*
500 ppm GA_3 #2	0.14 ± 0.06*	0.26 ± 0.14*
5 ppm ABA	0.30 ± 0.05	0.46 ± 0.11
25 ppm ABA	0.27 ± 0.09	0.58 ± 0.15
50 ppm ABA	0.31 ± 0.08	0.60 ± 0.16

* significantly lower than the tween-treated control, according to student's t-test ($p < 0.01$)

Table 4. Flavonoid composition in samples of juice from treated and control fruit (29–30 samples each). GA #1 are the individual fruit spray treatments, GA #2 are the whole tree spray treatments

treatment	flavonoid (mg/ml ± standard deviation)	
	narirutin	naringin
control 2.15	0.16 ± 0.05	0.45 ± 0.08
control 2.28	0.14 ± 0.04	0.39 ± 0.07
tween control	0.15 ± 0.06	0.39 ± 0.12
5 ppm GA_3 #1	0.11 ± 0.03	0.27 ± 0.06*
25 ppm GA_3 #1	0.12 ± 0.03	0.27 ± 0.04*
100 ppm GA_3 #1	0.10 ± 0.03	0.24 ± 0.06*
5 ppm GA_3 #2	0.10 ± 0.02	0.26 ± 0.05*
50 ppm GA_3 #2	0.07 ± 0.03*	0.18 ± 0.04*
500 ppm GA_3 #2	0.06 ± 0.02*	0.18 ± 0.05*
5 ppm ABA	0.17 ± 0.03	0.45 ± 0.07
25 ppm ABA	0.14 ± 0.02	0.43 ± 0.07
50 ppm ABA	0.20 ± 0.03	0.12 ± 0.12*

* significantly lower than the tween-treated control, according to student's t-test ($p < 0.01$)

olin paste [12]. In that study, application of GA_3 had a positive effect on juice quality by lowering the concentration of naringin while not significantly affecting the percent acid and soluble solid content. Treatment with lanolin paste GA_3 also lowered naringin concentrations in peel, which is especially important for commercial juice production as the extraction process often liberates naringin from the peel.

This study assessed whether or not the same effect could be achieved by spray applications of GA_3. Previous work had shown that GA_3 is effectively absorbed by fruit from both lanolin paste and spray applications [26]. The GA_3 is retained longer in lanolin applications, but spray applications were equally effective in getting GA_3 into the fruit. The absorption of GA_3 has been shown to be enhanced by the addition of wetting agents and surfactants such as Triton X-100 [25]. However, it has also been noted that the combination of GA_3 and surfactants can result in peel damage and discoloration [15]. Most commercial applications of GA_3 are done relatively late in the growing season, but in these experiments GA_3 was applied much earlier, and damage was less pronounced, with little or no leaf drop. GA_3-treated fruit were significantly larger than untreated or control fruit. The peel often had a mottled green color and it was generally thicker

than that of the control fruit. These peel characteristics may not be acceptable to fresh fruit consumers. However, GA_3-treated fruit could be used for juice production.

The overall characteristics of the juice obtained from GA_3-treated fruit were outstanding. Neither Brix nor acid percentage were substantially affected by the treatments. In nearly all cases, GA_3 treatments lowered the concentration of naringin in the juice. This was more or less proportional to the GA_3 concentration applied, as the greatest effect was achieved by higher concentrations of GA_3, while lower application levels, especially the 5 ppm GA_3 treatment, had variable effects.

Significantly, when an informal taste evaluation panel conducted at our lab compared juice from the 100 ppm GA_3 treated fruit, 500 ppm GA_3 treated fruit, and control fruit, all 10 members of our panel could discriminate among the three juices. They all placed the samples according to relative bitterness, untreated being the most bitter, the 100 ppm GA_3 juice having somewhat less bitterness, and the 500 ppm GA_3 juice being the least bitter.

Complete flavonoid analyses were run to determine if there were any interesting differences in both the amounts and types of flavonoids produced in the treated fruit. We did not find any significant amounts of new flavonoids produced nor was the production of any of the minor flavonoids examined enhanced.

ABA treatments had little effect on fruit size or juice characteristics. Testing ABA as a preharvest treatment was based on the observation that increased levels of ABA are associated with wounding in many plants [31, 33]. Therefore, ABA might alter the production of phytochemicals associated with defense mechanisms in the plant and thereby "immunize" them. From unpublished work in my lab on the effect of ABA on the induction of the accumulation of unique flavonoids in Mexican lime cell cultures, I thought the ABA might induce the formation of additional flavonoids in treated grapefruit. However, no new or different flavonoids were observed in the ABA-treated fruit. With the sensitive analytical techniques that are available today, induction of the accumulation of any additional or different flavonoids could be observed and quantified. In general, a slight increase in the flavonoid levels was observed in ABA-treated fruit.

Early GA_3 treatment lowered the concentration of all the flavonoids in the fruit, especially at higher levels. This re-enforces the theory that GA_3 treatments cause an overall enlargement of the fruit, rather than actually altering flavonoid biosynthesis. However, the variable effects on the other flavonoids in the fruit tissues might argue against a strict dilution effect. There may be some alteration of constitutive flavonoid biosynthesis by the PGRs.

Preharvest GA treatments applied just before color break on fully expanded fruit have been shown to be an effective means of enhancing resistance to Caribbean fruit fly [23, 38, 43]. Analysis of naringin concentrations in treated fruit in these previous experiments showed that the levels were lowered slightly. The experiments described in this paper show that earlier treatments with GA have a greater effect on lowering the naringin levels in fruit. It may be that this earlier treatment will also be effective in increasing the length of time fruit are resistant to fruit fly infestation.

The experiments reported in this paper were conducted with multiple sprays at high levels of compounds to insure that measurable effects were obtained. Further research should be done to see if a single GA_3 treatment at lower concentrations could also be effective in reducing naringin levels.

Acknowledgments

This research was conducted at the USDA, ARS, Fruit and Vegetable Chemistry Laboratory in Pasadena, which was closed by the USDA in 1994. The work was also funded in part by the Citrus Products Technical Committee. The author would like to thank Sadie Vannier for her technical assistance during the course of this work, the University of California Citrus Research Center and Agricultural Experiment Station in Riverside, California for the use of their trees, and Dr. Charles W. Coggins, Jr. for help with the spray application of the PGRs as well as helpful discussions regarding this project.

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